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Platelet Endothelial Cell Adhesion Molecule-1, a Putative Receptor for the Adhesion of *Streptococcus pneumoniae* to the Vascular Endothelium of the Blood-Brain Barrier

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The Gram-positive bacterium *Streptococcus pneumoniae* is the main causative agent of bacterial meningitis. *S. pneumoniae* is thought to invade the central nervous system via the bloodstream by crossing the vascular endothelium of the blood-brain barrier. The exact mechanism by which pneumococci cross endothelial cell barriers before meningitis develops is unknown. Here, we investigated the role of PECAM-1/CD31, one of the major endothelial cell adhesion molecules, in *S. pneumoniae* adhesion to vascular endothelium of the blood-brain barrier. Mice were intravenously infected with pneumococci and sacrificed at various time points to represent stages preceding meningitis. Immunofluorescent analysis of brain tissue of infected mice showed that pneumococci colocalized with PECAM-1. In human brain microvascular endothelial cells (HBMEC) incubated with *S. pneumoniae*, we observed a clear colocalization between PECAM-1 and pneumococci. Blocking of PECAM-1 reduced the adhesion of *S. pneumoniae* to endothelial cells *in vitro*, implying that PECAM-1 is involved in pneumococcal adhesion to the cells. Furthermore, using endothelial cell protein lysates, we demonstrated that *S. pneumoniae* physically binds to PECAM-1. Moreover, both *in vitro* and *in vivo* PECAM-1 colocalizes with the *S. pneumoniae* adhesion receptor pIgR. Lastly, immunoprecipitation experiments revealed that PECAM-1 can physically interact with pIgR. In summary, we show for the first time that blood-borne *S. pneumoniae* colocalizes with PECAM-1 expressed by brain microvascular endothelium and that, in addition, they colocalize with pIgR. We hypothesize that this interaction plays a role in pneumococcal binding to the blood-brain barrier vasculature prior to invasion into the brain.

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterial pathogen that causes life-threatening invasive diseases in humans, such as pneumonia and bacteremia. Every year, over a million people worldwide succumb to diseases caused by *S. pneumoniae* (1). Moreover, this bacterium is the most common causative agent of bacterial meningitis, an inflammation of the protective membranes covering the brain and spinal cord, collectively known as the meninges (2, 3). *S. pneumoniae* is thought to invade the brain mainly via the bloodstream by crossing the vascular endothelium of the blood-brain barrier, a specialized system of endothelial cells that protects the brain from harmful substances that are present in the bloodstream and supplies the brain with the required nutrients for its proper function (2, 3).

Meningeal pathogens, such as *S. pneumoniae* and *Neisseria meningitidis*, bind to receptors expressed on the plasma membrane of epithelial and endothelial cells, and through this binding, they can invade and translocate over human cell layers (4–6). The platelet-activating factor receptor (PAFR) has been described as one of the major receptors involved in the interaction between *S. pneumoniae* and endothelial cells (7–9). Blocking of PAFR leads to a significant reduction of pneumococcal adhesion to endothelial cells *in vitro*, and complete absence of PAFR leads to less invasive pneumococcal disease in mouse models (4, 7). However, we recently obtained data suggesting that a physical interaction between *S. pneumoniae* and PAFR is not likely to occur, as we did not observe colocalization between the receptor and the bacteria in the brain tissue of intravenously infected mice (10). Orihuela et al. showed that the laminin receptor can initiate the contact of *S. pneumoniae* with the brain vascular endothelium *in vivo* (11). The polyimmunoglobulin receptor (pIgR) can mediate binding of

pneumococci to the epithelium of the upper respiratory tract (5, 12), and we recently showed new evidence that pIgR is also implicated in binding of *S. pneumoniae* to brain endothelial cells (10). At the moment the extent of their contribution is unclear, and it remains to be established whether more receptors are involved in pneumococcal adherence to the blood-brain endothelium.

Platelet endothelial cell adhesion molecule-1 (PECAM-1; also known as CD31) is a panendothelial protein that is present in the intercellular junctions of the endothelial cells (13, 14). PECAM-1 is involved in leukocyte migration, angiogenesis, and integrin activation (13, 14). In particular, the involvement of PECAM-1 in leukocyte-endothelium interaction and leukocyte transendothelial migration makes PECAM-1 a key molecule in inflammation and neuroinflammation (15, 16). Recently, PECAM-1 was implicated in *Salmonella enterica* serovar Typhimurium infections (17), which raised the question of whether PECAM-1 also plays a role in

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TABLE 1 Antibody combinations used for immunofluorescent detection^a

Antigen	Antibody	
	Primary	Secondary
<i>S. pneumoniae</i>	Anti-capsule serotype 4 antibody (Statens Serum Institute, Copenhagen, Denmark), diluted 1:200 Antipneumococcal antiserum (immunization performed by Eurogentec, Maastricht, The Netherlands), diluted 1:50	Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen Life Technologies, Carlsbad, CA), diluted 1:500 Labeling with Alexa Fluor 350 (Zenon rabbit IgG labeling kit; Invitrogen Life Technologies)
Endothelial cells	DyLight 594-labeled <i>Lycopersicon esculentum</i> lectin (tomato lectin; Vector Laboratories, Burlingame, VT), diluted 1:200 Rat anti-mouse PECAM-1 antibody (BD Biosciences, Breda, The Netherlands), diluted 1:50 Mouse anti-human PECAM-1 antibody (Dako), diluted 1:50	Alexa Fluor 594 goat anti-rat antibody or Alexa Fluor 488 goat anti-rat antibody (Invitrogen Life Technologies), diluted 1:500 Alexa Fluor 594 goat anti-mouse antibody (Invitrogen Life Technologies), diluted 1:500
Mouse pIgR	Goat anti-mouse pIgR antibody (R&D Systems, Abingdon, United Kingdom) 1:50	Alexa Fluor 488 donkey anti-goat antibody (Invitrogen Life Technologies), diluted 1:500
Human pIgR	Goat anti-human pIgR antibody (R&D Systems, Abingdon, United Kingdom), diluted 1:50	Alexa Fluor 488 donkey anti-goat antibody (Invitrogen Life Technologies), diluted 1:500
α-Tubulin	Mouse anti-human α-tubulin (Sigma Aldrich), diluted 1:200	Alexa Fluor 594 goat anti-mouse antibody (Invitrogen Life Technologies), diluted 1:500

^a Mouse IgG (Innovative Research, Plymouth, MA), rat anti-human CD4 (AbD Serotec, Martinsried, Germany), and goat IgG (Santa Cruz Biotechnology, Dallas, TX) were used as IgG controls for mouse anti-human PECAM-1, rat anti-mouse PECAM-1, and goat anti-mouse/human pIgR, respectively, at the same dilutions as those for the primary antibodies. Cellular nuclei were stained with DAPI (diluted 1:5,000; Roche, Mannheim, Germany).

host-pneumococcal interactions. Accordingly, the aim of the present study was to investigate whether PECAM-1 plays a role in *S. pneumoniae* adhesion to the endothelium of the blood-brain barrier. To study this, BALB/c mice were intravenously infected with *S. pneumoniae* strain TIGR4 and sacrificed at different time points preceding meningitis (18), after which we assessed pneumococcal presence and localization in relation to the receptors studied. In addition, we investigated pneumococcal adhesion to endothelial cells *in vitro*. The immunofluorescent and confocal microscopic analyses of brain sections, in combination with *in vitro* blocking experiments and immunoprecipitation assays, show that PECAM-1 may well be a novel adhesion receptor for pneumococci that exerts its action in conjunction with pIgR.

MATERIALS AND METHODS

Cell lines, primary cells, and culture conditions. Human brain microvascular endothelial cells (HBMEC; obtained from K. S. Kim) and human umbilical vein endothelial cells (HUVEC; obtained from the Endothelial Cell Facility, UMCG) were cultivated as previously described (19, 20).

Bacterial strains and growth conditions. For the *in vivo* experiments, encapsulated *S. pneumoniae* serotype 4 strain TIGR4 (11, 18) was used. For the adhesion assays and the *in vitro* studies on physical interactions between bacteria and receptors, unencapsulated *S. pneumoniae* TIGR4 was used (21). The capsule impedes adhesion and invasion into host cells, yet it also has been shown that *S. pneumoniae*, in close interaction with host cells, loses its capsule (4, 21). This is why we, like others in the field (4, 8, 21), used unencapsulated pneumococci to study the *S. pneumoniae*-host cell interactions. The preparations of the challenge dose of encapsulated TIGR4 for the mouse experiments and of unencapsulated TIGR4 for the *in vitro* experiments were performed as previously described (18, 22).

Bacteremia-derived meningitis model. All experiments involving animals were performed with the prior approval of and in accordance with guidelines of the Institutional Animal Care and Use Committee of the University of Groningen (DEC no. 6152A). The bacteremia-derived meningitis model described by Orihuela et al. (11) was used as described before (18).

Antibodies and lectin. The antibodies used for immunofluorescent detection were diluted in sterile phosphate-buffered saline (PBS) with 5%

fetal calf serum (FCS) (Biochrom, Berlin, Germany). The antibody combinations are summarized in Table 1.

Immunofluorescent detection. Five-μm-thick brain/lung sections were fixed with acetone for 10 min. After 1 h of incubation with unencapsulated TIGR4, HBMEC and HUVEC were washed with PBS to remove nonadherent bacteria and fixed with 4% paraformaldehyde (Sigma-Aldrich) prior to starting the staining procedure.

After fixation, cells and tissue sections were incubated with primary antibody for 1 h at room temperature (RT). After washing with PBS, incubation with secondary antibody for 1 h at RT followed. To detect nuclei, incubation with 4',6-diamidino-2-phenylindole (DAPI) for 10 min at RT was performed, and then slides were washed with PBS and covered with Citifluor solution (Science Services, Munich, Germany). The slides were analyzed with a Leica DM5500B microscope, and images were recorded with a Leica DFC 360 FX camera. Before taking each image with the fluorescence microscope, the fluorescence signal in each channel was monitored with the Q-LUT function of the program Qwin (Leica Microsystems), which was used for image processing. The Q-LUT function shows the signal saturation levels within the areas studied; the over-saturated signal shows up in red, while the signal that is not oversaturated remains in grayscale. When the red signal was widespread in the field of view, the exposure and gain were reduced in order to minimize the over-saturated signal. The optimal setting was chosen for imaging in order to get a good overview of the PECAM-1 signal as a whole (in each figure). For confocal imaging, a Leica SP2 AOBs microscope was used. Immunofluorescent staining steps are summarized in Table 2.

Image processing. The TIFF images obtained with the 350-nm (blue)-, 488-nm (green)-, and 594-nm (red)-wavelength filters of the Leica DM5500B fluorescence microscope were merged using the color-merge channels function in ImageJ (23). The LEI z-stacks obtained with the Leica SP2 AOBs confocal microscope were merged through Imapis (Bitplane Scientific Software, Zurich, Switzerland). Brightness and contrast were adjusted with the ImageJ function image-adjust-brightness/contrast in such a way that (i) all images shown in Fig. 1 were adapted by the same adjustment of brightness and (ii) no information in each image has been obscured, eliminated, or misrepresented.

Colocalization and bacterial quantification analysis using ImageJ. The images representing the bacterial and PECAM-1 signals in HBMEC were analyzed with the ImageJ application named colocalization analysis

TABLE 2 Immunofluorescent detection scheme

Detection target	Incubation step		
	1	2	3
PECAM-1 and <i>S. pneumoniae</i> in mouse tissue and human cells	Mixture of anti-PECAM-1 and anti-capsule serotype 4 antibodies	For mouse tissue, mixture of Alexa Fluor 594 goat anti-rat and Alexa Fluor 488 goat anti-rabbit; for human cells, mixture of Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit	DAPI (dilution, 1:5,000)
PECAM-1, pIgR, and <i>S. pneumoniae</i> in mouse tissue and human cells	Mixture of anti-PECAM-1 and anti-pIgR antibodies	Mixture of Alexa Fluor 594 donkey anti-mouse and Alexa Fluor 488 donkey anti-goat antibodies	Anti-capsule serotype 4 antibody labeled with Alexa Fluor 350 (Zenon kit)

(23). White pixels were automatically generated on the areas of the bacterial signal that colocalized with the PECAM-1 signal. Using two separate images, the surface covered by bacteria was measured using the threshold function of ImageJ (23) by determining the area occupied by the 488-nm bacterial signal (total bacteria) in the one image and the white pixels of the bacteria colocalizing with receptors in the other.

Western blotting procedure. Human PECAM-1, pIgR (both diluted 1:200), and α -tubulin (diluted 1:1,000) were detected by Western blotting with the same antibodies that were used for immunofluorescence staining of human cells. For detection of p53 and F-actin, a mouse anti-p53 antibody (Abcam, Cambridge, United Kingdom) and a mouse anti-F-actin antibody (Merck Millipore, Billerica, United States), each diluted 1:1,000, were used. Proteins of the HBMEC and HUVEC lysates (prepared as described below) were separated by SDS-PAGE using NuPAGE gels (In-

vitrogen) and then blotted (75 min, 100 mA per gel) onto a nitrocellulose membrane (Protran, Schleicher & Schuell, Bath, United Kingdom). Incubation with primary antibodies was followed by incubation with the secondary antibodies IRDye 800 CW goat anti-mouse (LiCor Biosciences, Bad Homburg, Germany) for PECAM-1, p53, F-actin, and α -tubulin detection and IRDye 800 CW donkey anti-goat (LiCor Biosciences) for pIgR detection. All secondary antibodies were diluted 1:5,000.

Physical interaction of *S. pneumoniae* with endothelial proteins. The lysis buffer consisted of 50 mM Tris-HCl (Promega, Mannheim, Germany), pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS (all from Sigma-Aldrich), 1 mM EDTA (Merck Millipore, Billerica, MA), and 1 \times protease inhibitors (Roche, Mannheim, Germany). Lysis buffer (250 μ l) was added to confluent monolayers of HBMEC or HUVEC grown in T25 flasks (TPP, Trasadingen, Switzerland).

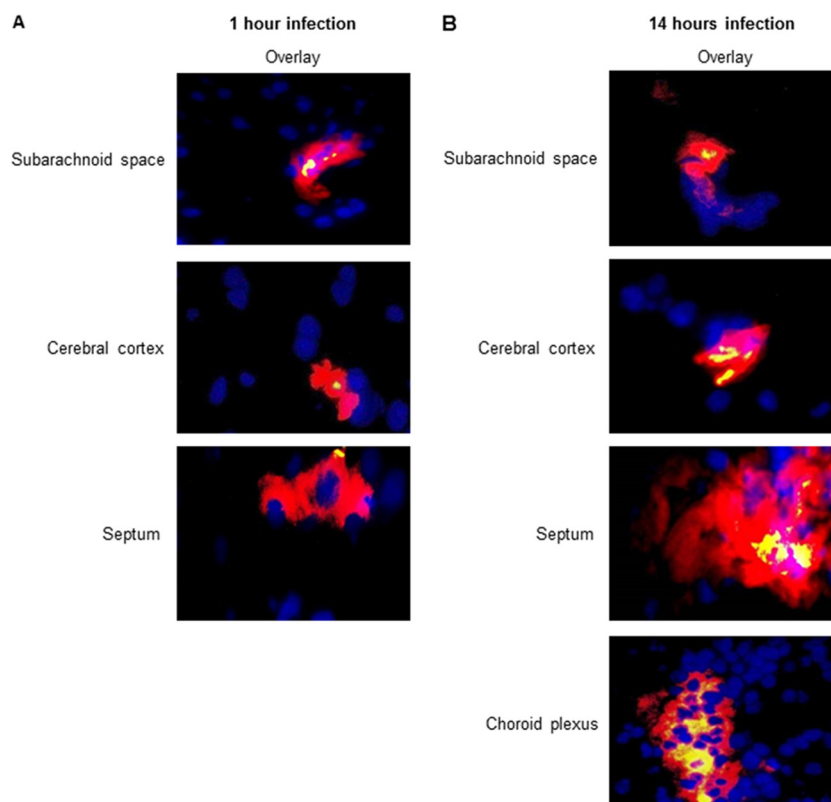


FIG 1 *In vivo* colocalization of *S. pneumoniae* and PECAM-1 on brain endothelium. Brain slides of mice systemically infected with *S. pneumoniae* were immunofluorescently stained for PECAM-1 (red), pneumococci (green), and cellular nuclei (blue). The images are representative for the situation in each brain compartment at 1 h (A) and 14 h (B) postbacterial challenge. At 1 h (and 3 h; see Fig. S1 in the supplemental material), no pneumococci were detected in the choroid plexus. For each time point of infection, 6 brain sections from 3 mice in each group were analyzed. The total magnification of subarachnoid space, septum, and choroid plexus was $\times 630$; the total magnification of the cerebral cortex was $\times 1,000$.

land). After 1 min of incubation at RT, monolayers were scraped with a cell scraper (NalgeNunc International, Rochester, United States), harvested, and centrifuged at 20,600 rpm for 15 min at 4°C. The supernatant fraction was used for further analyses. As a quality control, the lysates were separated by SDS-PAGE and proteins were visualized by Coomassie brilliant blue staining. A solution of approximately 10⁶ CFU of unencapsulated *S. pneumoniae* TIGR4 was prepared in sterile PBS. Fifty µl of HBMEC or HUVEC lysate was added to 50 µl of the bacterial solution, and the mixture was incubated at 4°C with gentle agitation for 1 h. The mixture of cell lysate and bacteria then was centrifuged at 20,600 rpm for 20 min at 4°C. The supernatant was removed and the bacterial pellet washed 2 times with 100 µl PBS. After washing, the pellet was incubated for 1 h at 4°C in 100 µl of a mixture of antipneumococcal antiserum and anti-PECAM-1 antibody in PBS. After washing in PBS, the bacterial pellet was resuspended in a mixture of Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse antibodies (diluted 1:500) in PBS. As negative controls, mouse anti-human α-tubulin/Alexa Fluor 488 goat anti-mouse antibodies were used. Finally, the bacterial pellet was resuspended in 100 µl of distilled water. Five µl was pipetted onto a microscope glass slide and covered with Citifluor solution. The slide then was analyzed with a Leica DM5500B fluorescence microscope, and images were recorded with a Leica DFC 360 FX camera.

Analysis of PECAM-1 expression in HBMEC and HUVEC. HBMEC and HUVEC were grown in 12-well plates (TPP) at 37°C with 5% CO₂ until confluence. In the case of bacterial adhesion, approximately 10⁷ CFU of *S. pneumoniae* TIGR4 was added per well. After 1 h of incubation at 37°C at 5% CO₂, cells were lysed (see “Physical interaction of *S. pneumoniae* with endothelial proteins”). HBMEC and HUVEC lysates were analyzed for PECAM-1 protein by Western blotting (see the Western blotting procedure described above). ImageJ was used to measure the density of bands of PECAM-1 and α-tubulin on Western blot membranes. Rectangles were drawn around PECAM-1 and α-tubulin bands. Using the Analyze/Gels/PlotLanes function, a graphical depiction of the intensity of pixels from the top of the rectangle to the bottom of the rectangle was generated, and the areas of each peak of PECAM-1 and α-tubulin bands were calculated. PECAM-1 signal was corrected for the α-tubulin control. Effects of bacterial adhesion on PECAM-1 protein expression were calculated by dividing the PECAM-1 signal after 1 h of incubation with pneumococci by the signal under normal conditions.

Pneumococcal adherence to endothelial cells. HBMEC and HUVEC were grown in 12-well plates (TPP) at 37°C with 5% CO₂ until confluence. After washing 2 times with PBS, receptor-specific antibodies and a mixture of anti-PECAM-1 plus anti-pIgR antibodies diluted in RPMI were added to each well to a final concentration of 50 µg/ml, and cells were incubated for 1 h at 37°C with 5% CO₂. As a control, we used HBMEC and HUVEC treated with mouse IgG (Innovative Research, Plymouth, MA), goat IgG (Santa Cruz Biotechnology), mouse IgG plus goat IgG mixture at 50 µg/ml, or just RPMI. After washing the cells with sterile PBS, 900 µl cell culture medium was added to each well and 100 µl of approximately 10⁷ CFU/ml of unencapsulated TIGR4 was added. After 1 h of incubation at 37°C under 5% CO₂, cells were treated with a 50/50 mix of 1% saponin (Sigma-Aldrich) and trypsin-EDTA (0.05% to 0.02%) (Gibco) and lysed. CFU were determined by plating serial dilutions of lysed cells on blood agar plates. The ratio of adherent bacteria was calculated by dividing the adherent bacteria by the total amount of bacteria in the well (adherent plus nonadherent bacteria).

Protein-protein interaction studies. Approximately 5 µg of anti-PECAM-1, anti-pIgR antibody, or mouse IgG was incubated with 15 µl of protein A/G PLUS agarose beads (Santa Cruz Biotechnology) at 4°C for 1 h under gentle agitation. Subsequently, to remove unbound antibody, the antibody-coated beads were centrifuged at 6,700 × g for 30 s at 4°C and washed with 500 µl of the lysis buffer used to make cell lysates. This procedure was repeated 3 times, and afterwards the pellet was resuspended in 50 µl of lysis buffer. Aliquots of cell lysates (~200 µg protein) were incubated with 10 µl of protein A/G PLUS agarose beads (Santa Cruz

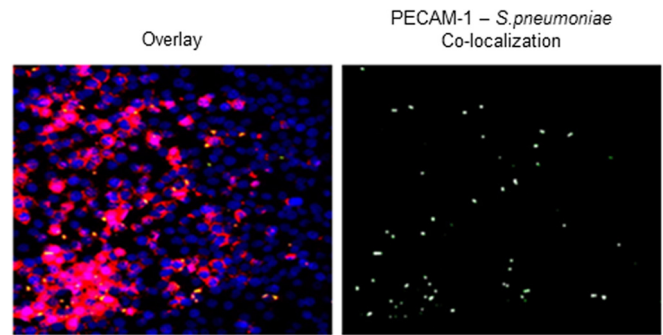


FIG 2 Colocalization of *S. pneumoniae* and PECAM-1 in HBMEC. Immunofluorescent detection of *S. pneumoniae* (green), PECAM-1 (red), and cellular nuclei (blue) in HBMEC after 1 h of incubation at 37°C with 10⁶ CFU pneumococci. Total magnification, ×630. On the right, the areas of the bacterial signal colocalized with the PECAM-1 signal appear in white (see “Colocalization and bacterial quantification analysis using ImageJ” in Materials and Methods).

Biotechnology) at 4°C for 1 h under gentle agitation. After centrifugation at 6,700 rpm for 30 s at 4°C, the supernatants consisting of cleared cell lysates were mixed with antibody-coated beads and the total mix was incubated at 4°C for 1 h under gentle agitation. Samples then were centrifuged at 8,000 rpm for 30 s at 4°C, and the pellets were washed with 500 µl of lysis buffer. This procedure was repeated 3 times. Lastly, the pellets were resuspended in 50 µl of lysis buffer and boiled at 95°C for 5 min. Proteins in the different samples were separated on NuPAGE gels (Invitrogen) and then blotted (as described above).

Statistical analysis. SPSS Statistics 20 (IBM) was used for the statistical analyses of the adherence assay results and the quantitative Western blot data. The independent-sample *t* test was used for the analysis of conditions in which two groups were compared for adherence assay results (blocking versus isotype control IgG and blocking versus no treatment) and for the analysis of Western blot data of PECAM-1 expression. In addition, analysis of variance (ANOVA) was used for multigroup comparison of the adherence assay data. The Mann-Whitney U test was used to test whether the reduction of adhesion obtained when PECAM-1 and pIgR were blocked at the same time was higher than the reduction when PECAM-1 and pIgR were blocked separately. Differences were considered significant when *P* < 0.05 (two-sided test).

RESULTS

***S. pneumoniae* colocalizes with PECAM-1 expressed by the vascular endothelium of the blood-brain barrier *in vivo* and by HBMEC *in vitro*.** Previously we have shown that, as soon as 1 h after intravenous infection, pneumococci adhere to the blood-brain endothelium. Since we observed significant spatiotemporal differences in the invasion of the brain (18), we investigated the possible colocalization of *S. pneumoniae* with PECAM-1 at all time points in all brain compartments during infection. These immunofluorescent analyses revealed that, at all time points of infection, more than 95% of adherent bacteria colocalized with PECAM-1 (Fig. 1A and B; also see Fig. S1A and B in the supplemental material). This observation was verified subsequently by confocal microscopy, which confirmed that *S. pneumoniae* was indeed colocalized with PECAM-1 (see Fig. S2). *In vitro*, colocalization analyses also showed that most pneumococci colocalized with PECAM-1 in HBMEC cells (Fig. 2). To check for possible bleed-through between the 488- and 593-nm channels, we also stained the HBMEC with anti-PECAM-1 antibody only, followed by Alexa Fluor goat anti-mouse 594 (red). Under this condition,

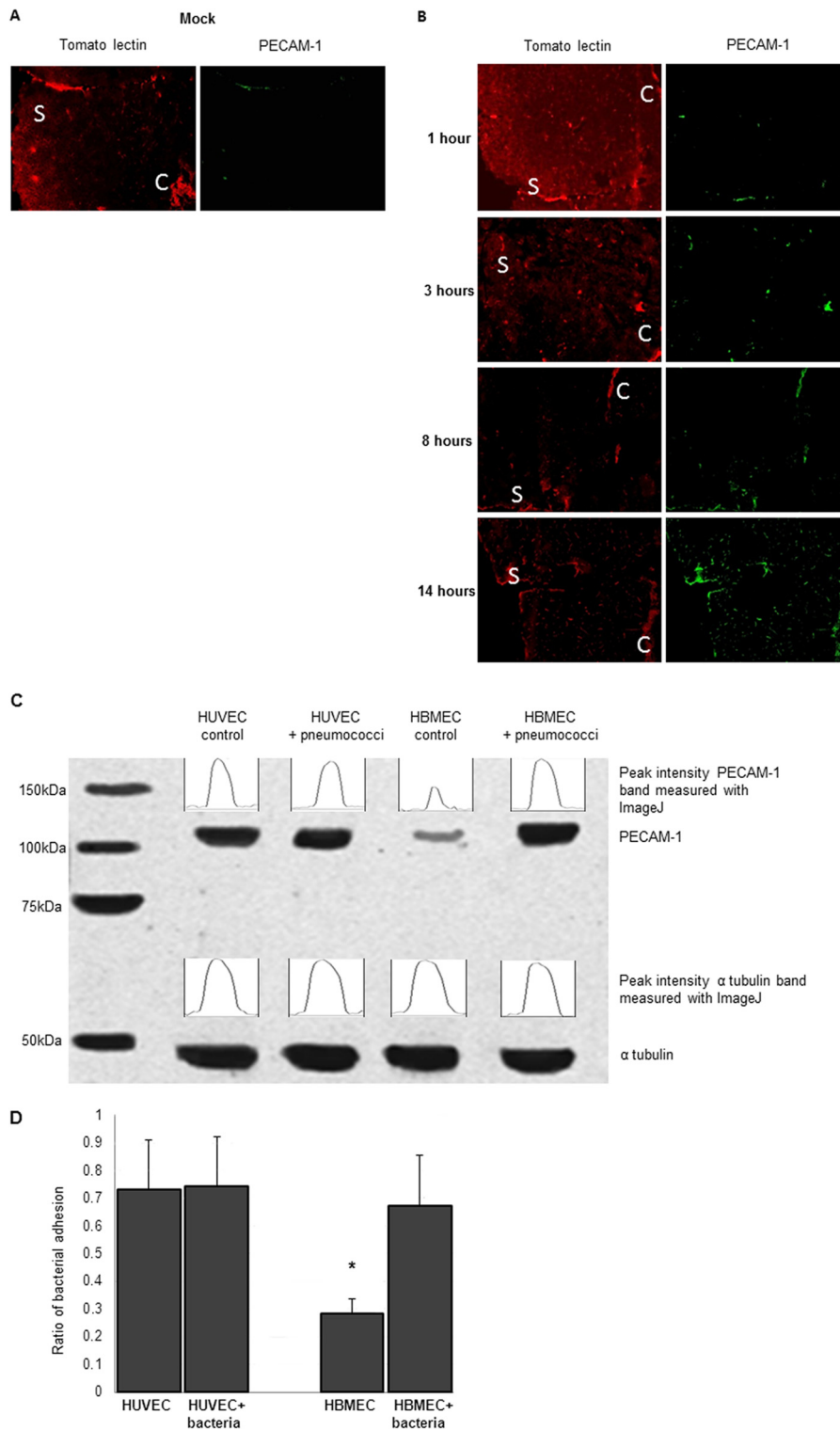


FIG 3 Increased PECAM-1 protein expression in the presence of pneumococci. (A and B) Immunofluorescent detection of tomato lectin (red) and PECAM-1 (green) in the brain of mock-treated mice (A) and *S. pneumoniae*-infected mice (B) at different time points postinfection. Total magnification, $\times 50$. Brains from 2 mock-treated and 3 *S. pneumoniae*-infected mice were analyzed, and 3 brain sections of each mouse were analyzed. The images are representative of all mice that have been analyzed. (C) Expression of PECAM-1 protein in HUVEC and HBMEC under normal conditions and after 1 h of incubation with *S. pneumoniae* as assessed by Western blotting. Data represent 3 biological replicates. The molecular masses of PECAM-1 and α -tubulin are approximately 120 kDa and 50 kDa, respectively. Peaks of intensity of PECAM-1 and α -tubulin signals generated with ImageJ are also shown. (D) Effects of bacterial adhesion on PECAM-1 expression. Relative PECAM-1 signal in HUVEC and HBMEC under normal conditions and after 1 h of incubation with *S. pneumoniae*. The y axis shows the ratios of ImageJ-determined peak intensities of tubulin-corrected PECAM-1 in the absence and presence of bacterial adhesion. *, $P < 0.05$ for HBMEC versus HBMEC plus bacteria.

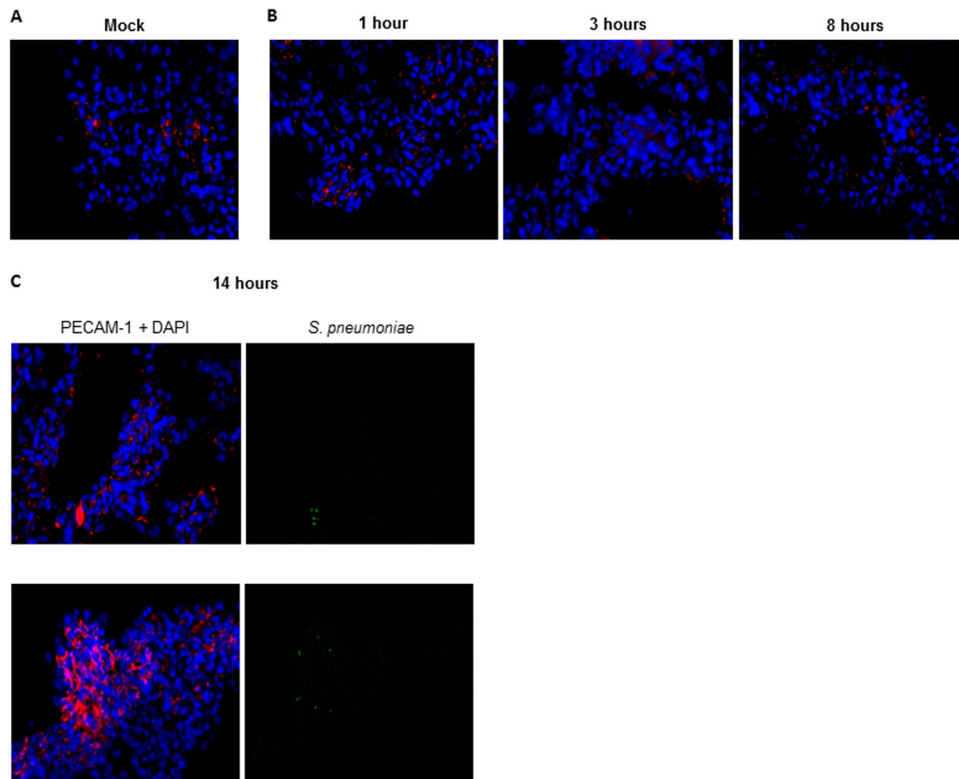


FIG 4 Specific induction of PECAM-1 in the presence of pneumococci in the lungs. Immunofluorescent detection of PECAM-1 (red) and cellular nuclei (blue) in lungs of mock-infected mice (A), during systemic infection with *S. pneumoniae* up to 8 h postchallenge (B), and in lungs of infected mice at 14 h postchallenge (C). No pneumococci were detected in lungs of infected mice up to 8 h postinfection (data not shown). Bacteria were occasionally detected in the lungs at 14 h postchallenge. Total magnification, $\times 400$. Lungs from 3 infected mice were analyzed, and 3 lung sections of each mouse were stained. The images show representative outcomes for each time point and condition.

no 488-nm (green) fluorescent signal was detected. We also stained HBMEC with antipneumococcal antiserum, followed by Alexa Fluor goat anti-rabbit 488 (green). In this case as well, no 594-nm (red) signal was detected (data not shown).

Our immunofluorescent analyses furthermore showed that in brain tissue of mock-treated, healthy mice, PECAM-1 signal was weak and patchy (Fig. 3A), while over the time course of *S. pneumoniae* infection the signal increased. In particular, it seemed that this increase in PECAM-1 signal was detectable in association with pneumococci or nearby areas where the bacteria were present (Fig. 3B). This also was observed in the subarachnoid space at 1 h, cerebral cortex at 8 h, and choroid plexus at 8 and 14 h (see Fig. S2 in the supplemental material). The part of the vasculature associated with and/or in close proximity to bacteria shows a high PECAM-1 signal, while where bacteria are absent near the vasculature, a low PECAM-1 signal is seen (see Fig. S2).

To investigate whether the presence of bacteria induces an increase in PECAM-1 protein expression, we analyzed by Western blotting PECAM-1 expression in HBMEC and HUVEC under normal conditions and after 1 h of incubation with pneumococci. Upon incubation with bacteria, the level of PECAM-1 in HBMEC significantly increased, while in HUVEC its levels did not change (Fig. 3C and D).

To confirm that the PECAM-1 protein levels increased in the presence of pneumococci and not because of inflammation caused by bacterial infection, we immunofluorescently stained

PECAM-1 in lung tissue. We previously showed that intravenous injection of *S. pneumoniae* TIGR4 causes a high influx of leukocytes in the lungs from 1 to 14 h postchallenge, which is a clear sign of inflammation (18). Between lungs of mock-treated mice and lungs of infected mice, no differences in PECAM-1 signal were observed up to 8 h postchallenge (Fig. 4A). Bacteria occasionally were detected in the lungs only at 14 h postchallenge (Fig. 4B), which was paralleled by a more intense PECAM-1 expression near the areas where bacteria were visible (Fig. 4B). Together, these results suggest that the presence of pneumococci induces PECAM-1 protein expression in the endothelium.

Blocking of PECAM-1 reduces adhesion of *S. pneumoniae* to endothelial cells. The immunofluorescent analysis showed colocalization between pneumococci and PECAM-1 on brain endothelium *in vivo* and on brain endothelial cells *in vitro*. The next step was to investigate whether PECAM-1 is functionally involved in pneumococcal adhesion to endothelial cells. Indeed, in HBMEC and HUVEC treated with anti-PECAM-1 antibody prior to incubation with pneumococci, bacterial adhesion was ~ 45 to 50% reduced compared to the level under control conditions (Fig. 5A and B). This shows that PECAM-1 is involved in the adherence of *S. pneumoniae* to endothelial cells *in vitro*.

Physical interaction between *S. pneumoniae* and PECAM-1 in endothelial cell lysates. After the observation that blocking of PECAM-1 with a specific antibody leads to a significant reduction of pneumococcal adhesion to endothelial cells, we investigated

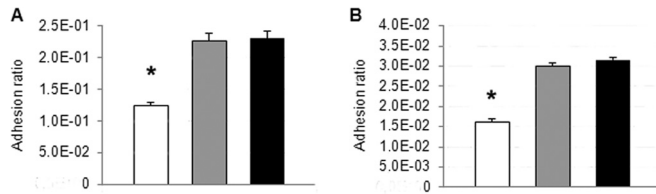


FIG 5 Blocking of PECAM-1 significantly reduces pneumococcal adhesion to human endothelial cells. Blocking of PECAM-1 with anti-PECAM-1 antibody (white bars) led to a significant reduction of pneumococcal adhesion to HBMEC (A) and HUVEC (B) compared to isotype IgG treatment (gray bars) and untreated HBMEC/HUVEC (black bars). Data represent averages from triplicate repeats of each condition plus standard deviations and are representative of three independent experiments. *, $P < 0.05$ for blocking of PECAM-1 versus IgG treatment or no treatment.

whether *S. pneumoniae* can physically interact with PECAM-1 present in lysates of endothelial cells. To this end, the bacteria were incubated with HBMEC or HUVEC lysates as described previously (10). In this assay, the bacteria were incubated with a lysate of cultured cells and collected by centrifugation. After extensive washing of the bacterial pellet, the bacteria were probed for the presence of bound eukaryotic proteins from the cell lysate using immunofluorescence. Indeed, a specific PECAM-1 signal was detected on the surface of pneumococci incubated with HBMEC or HUVEC (Fig. 6A), which indicates that PECAM-1 protein binds to pneumococci. In contrast, the control protein α -tubulin was not

detected on the pneumococcal cells (Fig. 6B), indicating that in this assay endothelial proteins generally do not become associated with the bacteria through nonspecific interactions. As a further control, bacteria which have not been incubated with endothelial cell lysates were stained with anti-human PECAM-1 antibody and no signal was detected (data not shown). Importantly, these results demonstrate that *S. pneumoniae* can physically interact with PECAM-1 protein that is present in lysates of HBMEC and HUVEC.

PECAM-1 interacts with pIgR, which may lead to the formation of a double receptor for the adhesion of *S. pneumoniae*. Our previous studies have shown that *S. pneumoniae* is closely associated with the pIgR receptor on brain endothelial cells and that *S. pneumoniae* association with pIgR can facilitate pneumococcal adherence to endothelium *in vitro* (10). In light of our present results, this suggests that pIgR and PECAM-1 should colocalize. Indeed, confocal imaging showed that PECAM-1 and pIgR are frequently colocalized in HBMEC and HUVEC when no bacteria are added (Fig. 7A and B). Furthermore, after incubation with *S. pneumoniae* for 1 h, pneumococci adherent to endothelial cells colocalized with both PECAM-1 and pIgR at the same time (Fig. 7C and D). Immunofluorescent analysis of brain tissues from noninfected mice also showed that PECAM-1 and pIgR were frequently colocalized in all brain compartments, as did analysis of brains from infected mice (Fig. 8A to C; also see Fig. S3A and B in the supplemental material). Lastly, most *S. pneumoniae* organisms colocalized with both PECAM-1 and pIgR (Fig. 8B and C; also see

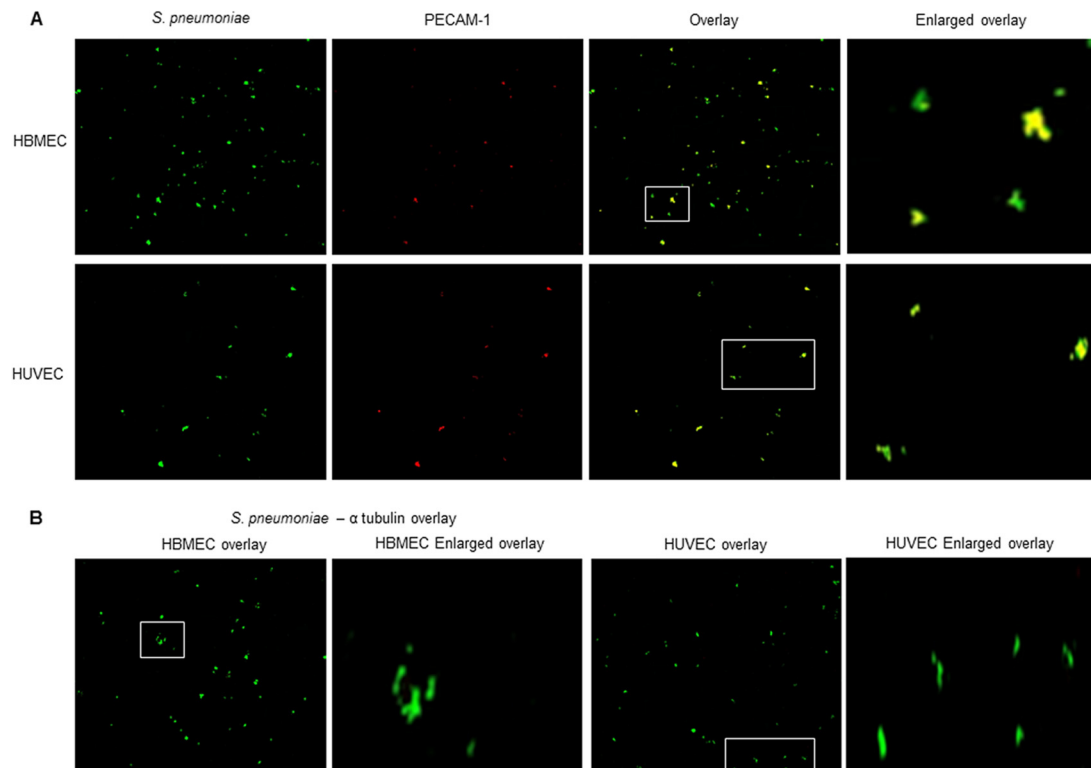


FIG 6 *S. pneumoniae* binds to PECAM-1 in HBMEC and HUVEC lysates. (A) Immunofluorescent detection of *S. pneumoniae* (green) and PECAM-1 (red) after pneumococci were incubated with HBMEC and HUVEC lysates. Total magnification, $\times 400$. The colocalization of the fluorescence in the overlay (yellow) indicates that PECAM-1 signal is associated with the pneumococci. (B) Immunofluorescent detection of *S. pneumoniae* (green) and α -tubulin (red) after pneumococci were incubated with HBMEC and HUVEC lysates. Total magnification, $\times 400$. The overlay indicates that the α -tubulin signal is not detectable on the pneumococci. Parts of the overlay images (within white rectangles) are enlarged and shown on the right of each figure.

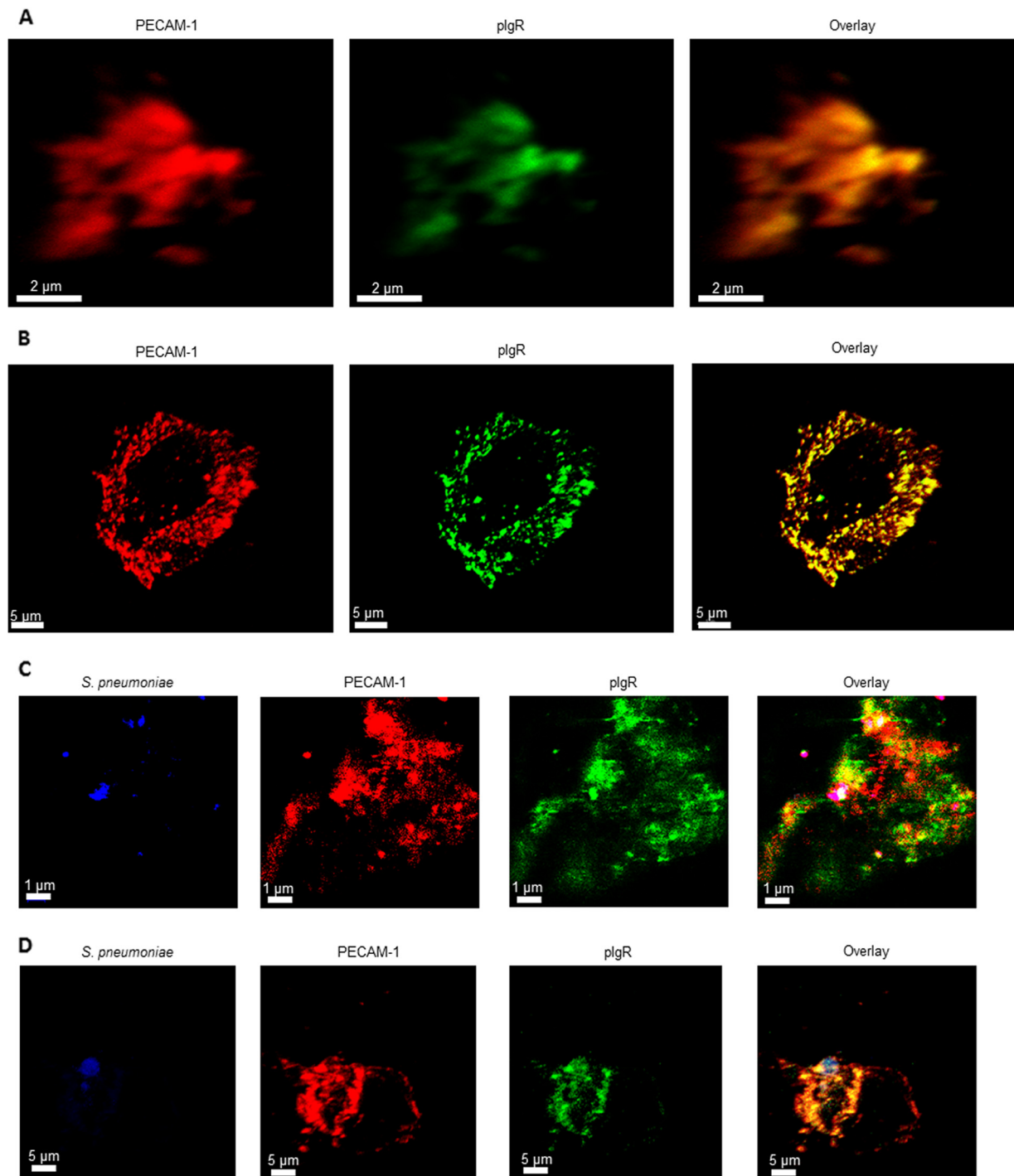


FIG 7 *In vitro*, endothelial PECAM-1, pIgR, and *S. pneumoniae* are colocalized. Confocal imaging of PECAM-1 and pIgR in HBMEC (A) and HUVEC (B) in the absence of bacteria and in HBMEC (C) and HUVEC (D) after a 1-h incubation with pneumococci. The overlay shows PECAM-1 being colocalized with pIgR. For each cell type, 10 cells were randomly selected for imaging. Each image shows one cell. The images are representative of the situation in the monolayer.

Fig. S3A and B), strongly supporting the model that PECAM-1 and pIgR both have a role in pneumococcal adherence to brain microvascular endothelial cells.

To determine whether PECAM-1 and pIgR interact with each other, we performed immunoprecipitation experiments in which we pulled down the one protein from HBMEC or HUVEC lysates

and detected the other one by Western blotting and *vice versa*. F-actin, which is known to interact with PECAM-1 (24), was used as a positive control. p53 was used as a negative control (Fig. 9A), since there is no evidence in the literature for a direct interaction between p53 and PECAM-1. Indeed, PECAM-1 and pIgR were coimmunoprecipitated with either antibody, which demonstrated

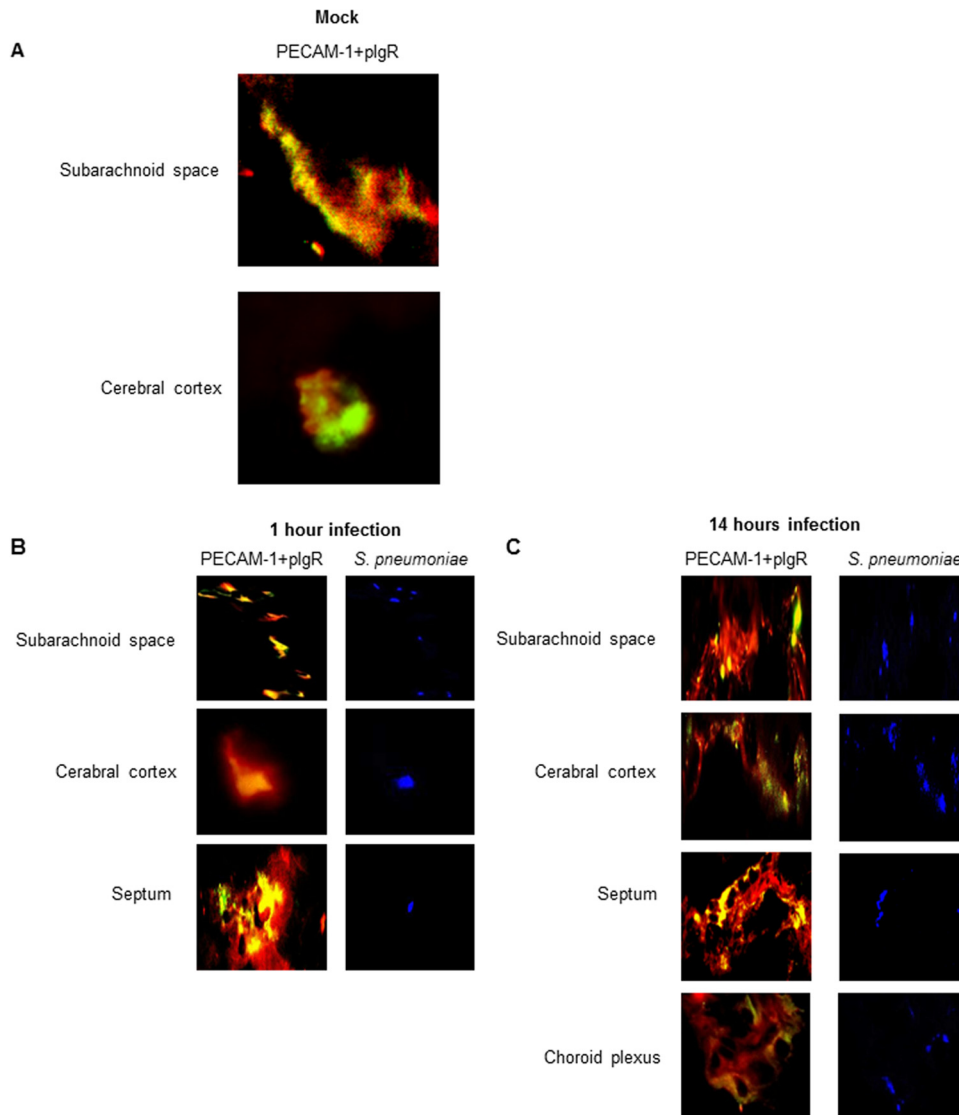


FIG 8 *In vivo*, PECAM-1, pIgR, and *S. pneumoniae* colocalize on brain microvascular endothelium. Immunofluorescent detection of PECAM-1 (red) and pIgR (green) in brain tissue of mock-treated mice (A) and of PECAM-1 (red), pIgR (green), and *S. pneumoniae* (blue) in brain tissue of infected mice at 1 h (B) and 14 h (C) postchallenge. The total magnification of the subarachnoid space, septum, and choroid plexus was $\times 630$; the total magnification of the cerebral cortex was $\times 1,000$. Brains from 2 mock-treated and 3 intravenously infected mice were analyzed, and 3 brain sections of each mouse were used for fluorescence staining. The images are representative of all mice within the groups that were analyzed.

that PECAM-1 and pIgR can physically interact in HBMEC and HUVEC lysates (Fig. 9A and B). Lastly, *in vitro* blocking data showed that the adhesion of *S. pneumoniae* to HBMEC and HUVEC was more extensively impaired when PECAM-1 and pIgR were simultaneously blocked than when either PECAM-1 or pIgR was blocked individually (Fig. 10). All of our data sets together suggest that PECAM-1 and pIgR cooperatively facilitate the adhesion of *S. pneumoniae* to endothelial cells *in vitro* and to brain microvascular endothelial cells *in vivo*.

DISCUSSION

The aim of this study was to investigate whether *S. pneumoniae* has the capacity to interact with PECAM-1 protein expressed by vascular endothelium of the blood-brain barrier. To study this, we mimicked the events preceding meningitis by intravenously in-

fecting mice with pneumococci and sacrificing them at various time points after challenge (18). In addition, we performed *in vitro* bacterial-endothelial adhesion assays. In the course of these analyses, we demonstrated that *S. pneumoniae* colocalizes with PECAM-1 *in vivo* and *in vitro*, and that PECAM-1 blocking significantly reduced the adhesion of *S. pneumoniae* to endothelial cells *in vitro*. In addition, we demonstrated PECAM-1/pIgR colocalization with bacteria on brain endothelial cells and showed that both interact with *S. pneumoniae* *in vitro* and are colocalized at the sites of bacterial adhesion *in vivo*. Furthermore, coimmunoprecipitation studies using endothelial cell extracts showed that PECAM-1 and pIgR can interact. Altogether, these observations imply that PECAM-1 and pIgR proteins can mediate the adhesion of *S. pneumoniae* to blood-brain barrier endothelium and possibly cofacilitate bacterial invasion into the brain.

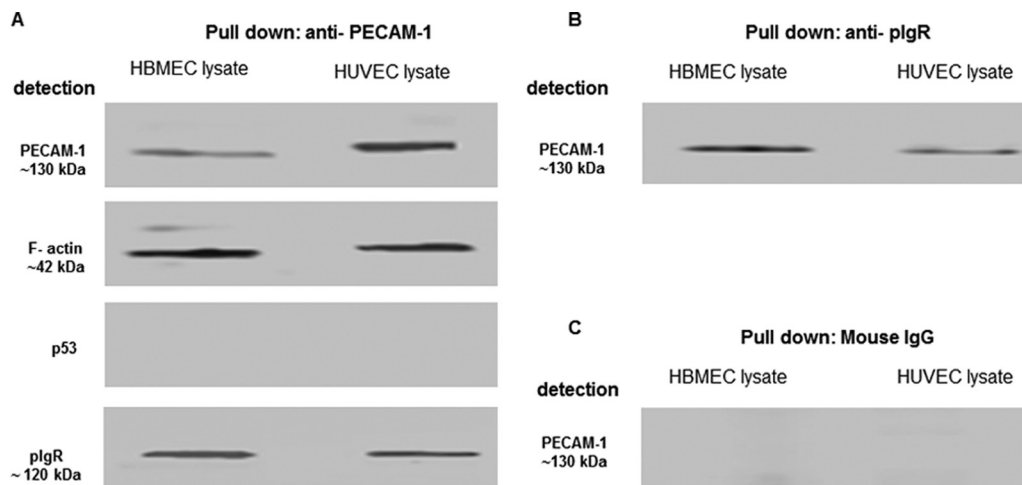


FIG 9 PECAM-1 interacts with pIgR in HBMEC and HUVEC lysates. (A) Immunoprecipitation of PECAM-1 and pIgR followed by Western blotting showed that PECAM-1 interacts with pIgR present in HBMEC and HUVEC lysates as well as with F-actin, while no interaction with p53 is detectable. (B) Immunoprecipitation of pIgR in HBMEC and HUVEC lysates followed by detection of PECAM-1. (C) Control immunoprecipitation with mouse IgG-coated beads does not show a PECAM-1 signal. Data represent 3 biological replicates.

Relatively little is known about PECAM-1 as a putative receptor for pathogens. PECAM-1^{-/-} mice were more resistant to gastrointestinal infection with *S. Typhimurium* than wild-type mice, resulting in lower bacterial loads in organs such as the liver and the spleen (17). Whole-bacterial-cell enzyme-linked immunosorbent assays revealed that human and murine PECAM-1 can bind to *S. Typhimurium*, indicating that transmembrane-expressed PECAM-1 is capable of binding to bacteria, which may have pathogenic consequences (17). In our study, *S. pneumoniae* was clearly shown to colocalize with PECAM-1 both *in vitro* and *in vivo*, and incubation of *S. pneumoniae* with endothelial cell lysates enabled PECAM-1 to associate with the bacteria. This implies that PECAM-1 also could serve as a receptor for *S. pneumoniae*, which may have consequences for the development of invasive pneumococcal disease (IPD). Unequivocal evidence for such a role needs

to be established in future studies employing, for example, endothelium-specific receptor knockout mice.

At present, we do not know which part(s) of *S. pneumoniae* is involved in its association with PECAM-1. PECAM-1 consists of 6 Ig-like domains. In the literature, it has been shown that specific components of *S. pneumoniae*, such as the major pilin RrgB, which forms the pilus shaft on the bacterium, and the keratin 10-binding domain of the adhesin PspA contain Ig-like domains (25, 26). Furthermore, it has been shown that Ig-like domains can react with a variety of different domain types; in particular, interactions with other Ig-like domains are the most frequent (27). Thus, one hypothesis is that the Ig-like domains present on *S. pneumoniae* and on PECAM-1 mediate the physical interaction between PECAM-1 and the bacteria.

As shown by immunofluorescence, PECAM-1 protein is expressed in the healthy mouse brain, although relatively weakly (Fig. 3A). Upon pneumococcal infection, the PECAM-1 signal increased, in particular when *S. pneumoniae* was associated with/in close proximity to the cells (Fig. 3B). Using lung tissues of the same mice, we showed that inflammation *per se* is not enough to induce the PECAM-1 signal and that the presence of the bacteria is needed. Together with the increased PECAM-1 expression by HBMEC upon bacterial adhesion, this implies that the induction of this receptor is a direct consequence of bacterial binding to the receptor. Additional experiments will be needed to further establish whether such a causal role between bacterial binding and (transcriptional or posttranscriptional) control of PECAM-1 expression exists and whether the induction has functional consequences.

After intravenous injection of 10⁷ CFU of *S. pneumoniae* TIGR4, the systemic numbers of bacteria were reduced by 10-fold at 1 h after the challenge (18), but as the disease progressed, the recoverable CFU in the blood increased, corroborating a previously reported study in a rabbit model (28). PECAM-1 is expressed by the endothelial cells of the entire vascular system (14), so it may be possible that this initial 10-fold drop in CFU in the blood within the first hour is the result of an immediate attach-

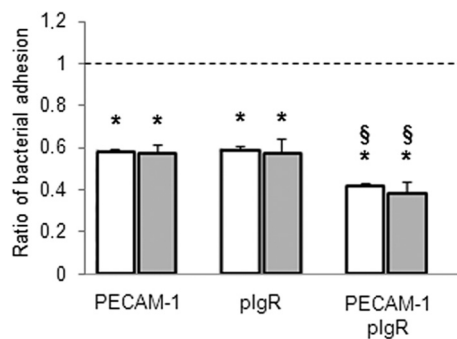


FIG 10 Blocking of PECAM-1 and pIgR reduces the pneumococcal adhesion to human endothelial cells to lower levels than blocking of the individual receptors. Blocking of PECAM-1, pIgR, and PECAM-1 plus pIgR in HBMEC (white columns) and HUVEC (gray columns) leads to a significant reduction of pneumococcal adhesion compared to that of controls treated with the respective isotype IgG. The adhesion ratio of controls was set at 1 and is indicated by the black dashed line. Treatment with specific receptor antibodies significantly reduced adhesion compared to that of controls in both HBMEC and HUVEC. *, $P < 0.05$. When both receptors are blocked at the same time, the reduction of pneumococcal adhesion is higher than that when blocking the individual receptors in both HBMEC and HUVEC. §, $P < 0.05$.

ment of bacteria to PECAM-1 in all blood vessels or in specific compartments of the body, which would sequester the bacteria. In principle, the immediate attachment of bacteria to the vasculatures may lead to bacterial invasion into these organs. However, *S. pneumoniae* is known mainly to cause pneumonia, bacteremia, otitis media, and meningitis. Therefore, it would be interesting to investigate in future studies whether blood-borne pneumococci also are able to translocate over the endothelium of other organs or whether they are protected from invasion by (unknown) molecular mechanisms that are lacking in the pathogenically preferred sites.

Receptor-mediated adhesion to human cells is a mechanism also described in meningeal invasion by *N. meningitidis*. It was reported that type IV pili of meningococci adhere first to a receptor, possibly PAFR (29), and afterwards to the β -adrenoceptor expressed on the cell membrane of HBMEC. These two receptors together allow meningococci to adhere to and traverse the endothelial cells, leading to invasion of the brain (6, 29). A similar receptor cooperation concept also may apply to *S. pneumoniae* adhesion to and invasion of endothelial cells of the blood-brain barrier. Here, we show that pIgR and PECAM-1 colocalize in the healthy murine brain and in the presence of infectious bacteria. Furthermore, blocking of both receptors with specific antibodies reduces pneumococcal adhesion approximately 20% more than blocking each receptor separately. Moreover, the immunoprecipitation analyses show that PECAM-1 and pIgR are capable of interacting with each other. Thus, it may well be that these two proteins form a double receptor on the plasma membrane of endothelial cells, which may have a functional consequence for bacterial adhesion to the endothelium and possibly also for further endothelial engagement in the *S. pneumoniae* translocation process.

To unequivocally demonstrate a role for these receptors *per se* in *S. pneumoniae* pathology, intravenous challenges with *S. pneumoniae* in PECAM-1^{-/-} mice and pIgR^{-/-} mice should be performed. In the case of a direct role, the prediction is that fewer bacteria would translocate the blood-brain barrier in the knockout mice; accordingly, meningitis symptoms should be reduced. Lastly, systemic challenges in mice lacking both PECAM-1 and pIgR should be performed to determine whether pneumococcal adhesion to the brain endothelium and disease symptoms are diminished to a greater extent in the double mutant mice than in mice lacking only one receptor. This would establish not only whether but also to what extent the two receptors cooperate and whether this has functional and pathological consequences.

In conclusion, based on the data presented, we propose that pneumococci in the bloodstream can bind PECAM-1 and/or pIgR expressed by brain microvascular endothelial cells, and that these two receptors on the plasma membrane of endothelial cells further facilitate the bacterial adhesion process. Additional experiments are needed to shed light on the functional role of both receptors in facilitating *S. pneumoniae* invasion into the brain *in vivo* and to answer the question of whether their blockade has any therapeutic advantage for patients with bacteremia and meningitis.

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